HYBRIDIZATION OF CULTIVATED SUNFLOWER *HELIANTHUS ANNUUS* WITH WILD ANNUAL SPECIES *HELIANTHUS BOLANDERI*, *HELIANTHUS NEGLECTUS,* AND *HELIANTHUS PETIOLARIS*

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Abstract

The species *H. bolanderi* Gray*, H. neglectus* Heiser and *H. petiolaris* ssp. *petiolaris* Nuttall were hybridized with the cultivated sunflower *H. annuus* L. The crossability rate was comparatively high. Seeds and hybrid plants were obtained from both directions of crossing. The F1 plants had an intermediate type of heritability, but they strongly resembled the wild parent for the most important biomorphological characters. The hybrid nature of individual F1 plants was confirmed through cytological and RAPD investigations. As a result of self-pollination, sib-pollination of the F1 plants and backcrossing to cultivated sunflower, F2, BC1, third and fourth hybrid progenies were obtained. It was established that the three species carried *Rf* genes for CMS PET1. Some of the obtained hybrid forms were included in a program for developing lines for heterosis breeding in sunflower.

Introduction

There are numerous and various investigations on the participation of annual species in hybridization with cultivated sunflower and the transfer of useful characters to cultivated sunflower (Satsyperov, 1916; Heiser, 1949; Putt and Sackston, 1957, 1963; Pustovoit, 1960; Leclercq, 1969; Pustovoit, 1975; Morized et al., 1984; Jan and Chandler, 1985; Chandler et al., 1986; Jan et al., 1989; Vannozzi et al., 1990; Atlagic et al., 1995; Christov et al., 1996). A large part of the results obtained have theoretical and applied significance. Besides proving the resemblance degree between the annual species and cultivated sunflower and describing the new developed plants, by using hybridization, important economic qualities and properties were transferred into cultivated sunflower, such as resistance to diseases and pests, drought tolerance, new sources of CMS and Rf genes, etc. (Satsyperov, 1916; Putt and Sackston, 1957 and 1963; Pustovoit, 1960; Leclercq, 1969; Morized et al., 1984; Jan and Chandler, 1985; Vranceanu et al., 1986; Serieys and Vincourt, 1987; Vannozzi et al., 1990, Christov et al., 1996; Christov, 1999).

This study presents results from the hybridization of cultivated sunflower (*H. annuus)* with three annual *Helianthus* species.

Materials and Methods

The investigation encompassed the period 1999-2002. It included the cultivated sunflower, *H. annuus,* and the annual species *H. bolanderi*, *H. neglectus* and *H. petiolaris* ssp. *petiolaris*. Cultivated sunflower was represented by two varieties, Peredovik and VNIIMK 6540, and by four lines, 2607, 6075, 6116, and HA 89, and the wild annual species accession *H. bolanderi* E 009, *H. neglectus* E 017, *H. petiolaris* E 021 and *H. petiolaris* E037 (Table 1).

Table 1. Some characteristics of annual species from genus *Helianthus* during 2000.

Species	Plant height (cm)	Leaf length (cm)	Leaf width (cm)	Head diameter (cm)	Bracts	Ray flowers	Tubular florets	1000-seed weight (g)
H .bolanderi E 009	150	14	11	2.8	31	19	175-224	5.2
H.. neglectus E 017	200	13	12.3	2.9	35	27	154-191	5.7
H. petiolaris E 021	200	11.5	7.7	3.7	39	23	189-240	6.1
H. petiolaris E 037	175	12.7	7	2.8	36	21	181-224	5.9
L HA 89	110	26.8	25	22	52	36	1338	57.6
cv. Peredovik	210	43	40	27	79	61	1785	74.1

Hybridization was carried out through reciprocal crosses realized under field conditions. The sterile analogues of lines 6075 and HA 89 were used as the female parent of the cultivated sunflower in direct crosses. Isolated sterile inflorescences of lines 6075 A and HA 89A were pollinated directly from the inflorescences of *H. bolanderi*, *H. neglectus* and *H. petiolaris*, which were excised from the plants before applying the pollen. In the reciprocal crosses, wild species x cultivated sunflower, the florets in the inflorescences of the wild species were manually emasculated and were pollinated with pollen from a single line or with mixed pollen from varieties and lines. Hybrid plants were grown under field conditions. Regular phenological observations were made during vegetation. Biometric measurements and description of the main morphologic characters and biologic peculiarities of the F_1 hybrids were performed. Similar investigations were carried out with the next hybrid generations as well. To obtain F2 and BC1, selfing, sib-pollination (re-pollination of F1 plants with similar plants from the same progeny) and backcrossing of F1 to cultural sunflower were made. Backcrossing was also carried out to obtain BC2 to overcome undesirable characters.

Cytological investigations were carried out on the meiosis of pollen mother cells (PMC) according to Georgieva-Todorova, 1976 and Atlagic, 1990. To investigate the meiosis and the chromosome associations in PMC, whole flower buds were taken at different stages from the development of the wild species *H. bolanderi*, the cultivated sunflower *H. annuus* and the F1 hybrid of *H. annuus* x *H. bolanderi*. Chromosome behavior was studied mainly in diakinesis, metaphase I, anaphase I and telophase II. Pollen viability was determined by a standard methodology (Owczarzak, 1952).

RAPD technique was used to determine the hybrid nature of the newly developed F1 sunflower forms. Genome DNA was isolated from the youngest sunflower leaves by the method of Dellaporta et al. (1983) with modifications of the AgroBioInstitute in Sofia. Kits for PCR analyses (Ready To Go PCR Beads, Amersham Pharmacia Biotech, Inc.) were used. For amplification of random DNA sequence, RAPD primers were involved (Operon

Technologies, USA, 10-mer): OPA-01, OPA-02, OPB-01, OPB-02 and OPB-07. The amplified products were separated by electrophoresis and were visualized on 2% agarose gel.

Purposeful selection was being carried out as early as F1. The investigations on disease resistance of the hybrids were done at the Dobroudja Agricultural Institute. Oil content in seed was studied. Female fertility of the plants was determined by the amount of seeds obtained after free pollination, and 1000-seed weight by measuring three samples, each of 50 seeds. Presence of sources of fertility restorer genes for CMS PET-1 was also registered.

Results and Discussion

Crossability of Cultivated Sunflower. Cultivated *H. annuus* was crossed with the annual species *H. bolanderi, H. neglectus,* and *H. petiolaris.*

Table 2. Crossability of cultivated sunflower *H. annuus* and wild annual *Helianthus* species (1999-2002).

Crosses	Pollinated inflorescences	Inflorescences with seed		Seed set		Hybrid plants	
		n	%	m.n.	%	n	%
H. annuus x *H. bolanderi*	8	6	75.0	108	8.1	319	49.2
H. bolanderi x *H. annuus*	12	7	58.3	2	10.6	37	25.2
H. annuus x *H. neglectus*	5	4	80.0	102	7.6	263	64.5
H. neglectus x *H. annuus*	8	4	50.0	14	8.1	18	32.1
H. annuus x *H. petiolaris*	12	8	66.7	91	6.8	431	59.2
H. petiolaris x *H. annuus*	11	6	54.5	15	7.4	21	23.1

The analysis of the results presented in Table 2 shows that the annual species *H. bolanderi, H. neglectus* and *H. petiolaris* can be crossed to cultivated sunflower. The crossability rate was comparatively high. Seed and hybrid plants were obtained from both directions of crossing. Seed set per cultivated sunflower inflorescence (lines HA 89A and 6075A) after compulsory pollination with pollen from the wild species was low, from 6.8 to 8.1%; after free pollination of these lines the seed set was significantly higher, 71.5% and 68.6%, respectively. The results were similar in the reciprocal crosses, as well. Their seed set was within the range from 7.4% to 10.6%. For *H. bolanderi, H. neglectus* and *H. petiolaris* a seed set of 65.3%, 40.7% and 46.4%, respectively, was registered.

The comparison of the results from the crossing of cultural sunflower to the annual species *H. bolanderi, H. neglectus* and *H. petiolaris* showed that pollination at both directions of crossing was not complete and was probably due to genetic differences between the wild annual species and cultivaated sunflower. Similar differences were observed in the results from the development of the seeds in F1 plants. The number of the obtained hybrid plants was lower than that of seeds. The percent of germinating hybrid plants was within the range of 23.1% to 64.5%. A higher percent of hybrid plants were produced in the cases when cultivated sunflower was used as a female parent. In the reciprocal crosses the percent of

hybrid plants was almost twice as low. There were differences in the percent of developed hybrid plants, though not significant, also according to the annual species included in the crosses.

Characterization of the Hybrid Progenies. Biomorphological characterization of F1 hybrid plants obtained from crosses of *H. annuus* with the three wild species were of intermediate type of heritability, but they strongly resembled the wild species for the most important biomorphological characters, even in those cases when they were used as the male parents. These characters were stem branches, peduncle length, and anthocyanin coloration of petioles, leaf veins and other characters of the whole plant.

All plants from the two-way crossing in the three groups of hybrids had erect and branched stems. The plants from the direct crosses reached a height of up to 150 to 160 cm, and those from the reciprocal crosses up to 170 cm, individual plants from the cross *H. neglectus* x *H. annuus* being as tall as 195 cm (Table 3). The stems were colored green, with anthocyanin spots, and were covered with coarse and sharp hairs. In the greater part of F_1 plants from both types of crosses, the central stem was higher than the branches and the central inflorescence was larger than that on the branches. In the direct crosses, 7 to 19 branches were registered, and in the reciprocal ones, 13 to 31 branches. In some plants from direct crosses the branches reached second class, and in the reciprocal crosses- third class.

Leaves were colored green. Their shape was similar to that of cultivated sunflower, but their size was smaller and the serration was different. Petioles had anthocyanin spots and were covered with short and coarse hairs. Leaf length varied from 12 to 16 cm.

An intermediate heritability type was observed in the size of inflorescence, the number of tubular florets and seed size and weight. The bracts were larger. The tubular florets and the stigmas were colored in light red or dark purple, and pollen and ray florets were dark yellow. The number of tubular florets in the central inflorescences varied from 293 to 424, and that of ray florets, from 19 to 25. Pollen viability was within the range of 26.1 to 95.9 %. Seed shelling began before full maturation of the head. Seed color was from pale gray-brownish to dark brown.

The vegetation period of the hybrids was shorter than that of the wild species. Differences were observed according to the direction of crossing. The hybrids which had cultivated sunflower as in mother form had shorter vegetation. Maturation of the main head began 5-10 days earlier than the cultivated parent.

Differences were also observed with regard to plant fertility. The highest amount of seeds was obtained after free pollination, followed by backcrossing with pollen from cultivated sunflower; the lowest seed number was produced after selfing of the inflorescences. The percent of inflorescence insemination was comparatively low. It was 0 in almost all selfed plants from the reciprocal crosses. In the case when the sterile analogues of lines 6075 and HA 89 were used as the mother form, fertile and male sterile plants were produced. The presence of fertile F1 plants (with tubular florets shedding pollen) showed that the species *H. bolanderi, H. neglectus* and *H. petiolaris* carried fertility restoration genes for CMS PET1. In this case only fertile plants were selected for selfing. Their feertilization percent varied from 1.26 to 19.22%.

Table 3. Characterization of F1 developed after crossing cultivated sunflower to the wild species *H. bolanderi, H. neglectus* and *H. petiolaris* (2000).

No, Hybrid, Generation	Plant height (cm)	Head diameter (cm)	1000-Seed weight (g)	Oil (%)	Vegetation period (days)
101, HA 89 A x *H. bolanderi*	140	9	28.9	34.8	115*
100. *H. bolanderi* x l6116	165	6	30.5	-	131*
23, 6075 A x *H. neglectus*	140	10	27.5	35.7	117*
361, *H. neglectus* x к. с.	175	8	28.1	36.4	133*
118. HA 89 A x *H. petiolaris*	135	7	24.5	31.5	113*
119. 6075 A x *H. petiolaris*	145	8	32.0	37.3	114*
111. *H. petiolaris* x к. с.	130	6	29.2	-	127*
L - HA 89	110	22	57.6	49.8	114
L - 6116	140	22	54.2	45.1	109
L 6075	100	21	56.4	43.2	112
Cv. Peredovik	210	27	74.1	49.9	122
H. petiolaris E 021	200	3.7	6.1	38.5	155*
H. petiolaris E 037	175	2.8	5.9	34.8	150*
H. neglectus	200	2.9	5.7	35.9	160*
H. bolanderi	150	2.8	5.2	34.3	162*

*Branched plants

Cytological Characterization. Characterization of F1 plants from the cross *H. annuus* x *H. bolanderi* showed a reduction of division of the PMC in the F1 plants with some deviations. Cells with 17 bivalents (i.e., chromosomes conjugated completely, the greater part of them being open), cells with 13-15 bivalents and 1-2 quadrivalents (ring and chain) (5.5%) and cells with 15-16 bivalents and 2-4 univalents (1.8%) were registered in diakinesis. The presence of complex configurations implied differences between the parental genomes in a

reciprocal translocation, and the presence of univalents was an indication of incomplete genome homology. Table 4 gives the meiotic analysis of the parental forms and the F1 hybrid. Single cells with non-included chromosomes were marked in metaphase I and anaphase I, which was a prerequisite for the occurrence of micronuclei and polyads. The formation of normal tetrads was observed during telophase II.

Table 4. Meiosis in the parental forms *H. annuus, H. bolanderi,* and F_1 hybrids in diakinesis.

A. Chromosome associations: variation and mean number per cell.

Material	Total number of cells	Bivalents				Univalents	Quadrivalents
		closed		open			
		from to	mean	from to	mean		
H. annuus x *H. bolanderi*	47	1-6	3.68	9-16	12.96	0 - 4	0 - 2
H. bolanderi	27	6-13	9.20	4-11	7.80	0	0
H. annuus	50	3-8	6.20	9-14	10.80	0	0

B. Meiotic analysis

Material	Mean frequency of bivalents per cell	Mean frequency of chiasmata per cell	Mean frequency of chiasmata per bivalent
H. annuus x *H. bolanderi*	3.68	20.68	1.23
H. bolanderi	9.20	26.18	1.54
H. annuus	6.20	23.20	1.36

The analysis of PMC from F plants established the formation of configurations different from those at regular meiosis in the parental PMC. This implied the existence of structural differences in the chromosomes of the parental genomes. The introduction of genes from the species *H. bolanderi* to cultivated sunflower showed a reduction of the normal pollen viability and lower female fertility of plants, which may be affected by other factors as well.

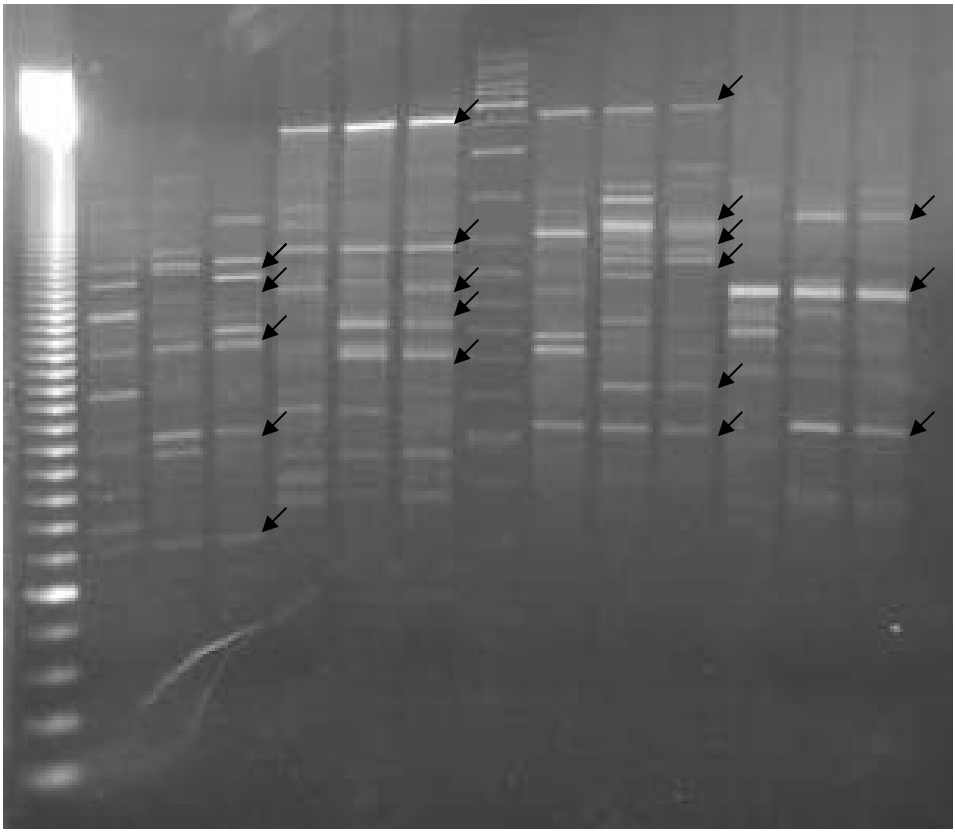
RAPD Analysis. F1 plants from the cross *H. annuus* x *H. neglectus* were investigated using five primers for amplification of genomic DNA from the parental species and F1 generation. The analysis was carried out on those fragments that were very visible. The comparison of the amplification profiles was based on the presence or absence of the fragments and the homology in their sizes.

Primers OPA-01, OPA-02, OPB-01 and OPB-07 (Figure 1) allowed the amplification of three specific fragments for the wild species and the hybrid, and nine specific fragments for the cultivated species and the hybrid. Seven bands were represented in the three genotypes.

Primer OPB-02 (Figure 2) allowed the amplification of specific fragments for one of the parents and the hybrid, and fragments that were common for the three genotypes. In the F1 genotype the form had two ands specific only for the genotype of cultural sunflower, and two bands specific only for the genotype of the wild species, as well as one band represented in all three genotypes. Out of the five primers used for amplification of the parents and the hybrid, two (OPB-01 and OPB-07) did not show specific fragments for the wild species and the hybrid.

The results reveal that there is polymorphism in the amplification PCR profiles of H. *neglectus, H. annuus* and *H. annuus* x *H. neglectus*, i.e., the RAPD analysis confirmed the

hybrid nature of the F1 material obtained from the crossing of the species *H. annuus* and *H. neglectus*. Furthermore, the electrophoregram of the three genotypes indicated introgression of species *H. neglectus* in the hybrid progeny.



1. *H. neglectus* - E 017; 2. *H. annuus* - l 6075A; and 3. F_1 - *H. annuus* x *H. neglectus.*

Figure 1. Electrophoregram of amplification profiles of the three genotypes with OPA-01, OPA-02, OPB-01 and OPB-07 primers.

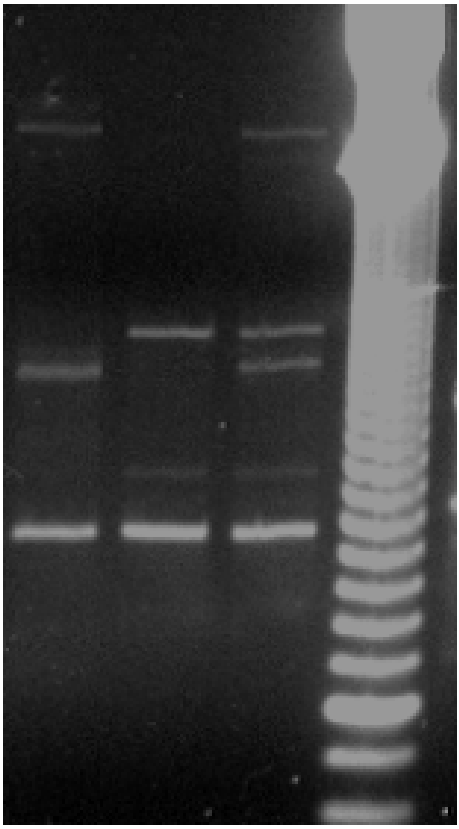


Figure 2. Electrophoregram of amplification profiles of the three genotypes with OPB-02 primer.

Characterization of Second and Later Generations. The second hybrid generation (F2 and BC2) was characterized with higher variability of forms. After re-pollination (sib-pollination) of the F1 plants of intermediate heritability type with similar plants from the same generation, segregation of branching, anthocyanin pigmentation, vegetation and many other characters were observed in the F2. All plants had branches, the type of branching being different and with different number of branches. There were single plants with deformed branches. Plant height was within the range of 125to 270 cm. Leaf size varied. The central inflorescences of all plants were normally developed, from 6 to 17 cm large. Seeds were colored in gray-brown to dark purple. Plant height from 140 to 195 cm was registered in the BC1 progenies. All plants had branches, but some of them had just a few. In other plants branches appeared on the main inflorescence after the end of anthesis. These plants had larger central heads, reaching up to 20 cm.

Plants without branches or with single branches were registered among the variability of forms in third hybrid generation. The size of the central inflorescence varied from 5 to 23 cm. Seeds were of different size, smaller or larger, colored in gray-black, black, dark brown-

purple or parti-colored. Oil content in seed was within the range from 34.6 to 55.1 %. Some of the plants combined many useful characters such as high oil content in seed and kernel, lack of anthocyanin color of peel and kernel coat, seed compactness, suitable 1000-seed weight of the forms with *Rf* genes, etc. Seeds with such characteristics were selected to produce the fourth generation. In the selection of plants the fact that from the forms obtained from the direct crosses restorer lines would be developed (R lines) possessing branched stems, and from the reciprocal crosses both branched and non-branched lines would be produced, was taken in account.

In the plants from the fourth hybrid generation variability was also observed, but there was greater difference between the plants from the individual numbers. A large part of the branched plants originating from the same number were similar by phenotype. The number of plants which lack of anthocyanin color or almost unobtrusive coloration was greater. Seeds were different in size and were mainly gray-black and black in color. Single plants had dark brown or anthocyanin-black seeds. Oil content in seed varied from 42.5 to 51.8 %. Some of the hybrid forms developed in third and fourth generation demonstrated resistance to downy mildew and Phomopsis and had high oil content in the seed. They were included in a program for developing of B and R lines for heterosis breeding in sunflower.

References

Atlagic, J., D. Skoric, B. Dozet and R. Marinkovic. 1995. Results of using interspecific hybridization in sunflower breeding. Selekcija I Semenarstvo. 2(1):63- 70.

Chandler, J. M., Chao-Chien Jan, and B. H. Beard. 1986. Chromosomal differentiation among the annual *Helianthu*s species. Systematic Botany. 11(2):354 - 371.

Christov, M. 1999. Production of new CMS sources in sunflower. Helia. 22(31):1-12.

Christov, M., P. Shindrova and V. Entcheva. 1996. Transfer of new characters from wild *Helianthus* species to cultivated sunflower. Genet. a Slecht. 32(4): 275-286.

Heiser, C. B. 1949. Study in the evolution of the sunflower species *H. annuus* and *H. bolanderi*. Univ. California Publ. Bot. 23:157-196.

Jan, C. C. and J. M. Chandler. 1985. Transfer of Powdery Mildew Resistance from *Helianthus debilis* Nutt to Cultivated Sunflower. Crop Science. 25:664-666.

Jan C. C. and J. M. Chandler. 1989. Sunflower interspecific hybrids and amphiploids of *Helianthus annuus* x *H. bolanderi*. Crop Science. 29:643-646.

Leclerc, P. 1969. Une sterile male cytoplasmique chez le tournesol. Ann. Amelior Plant. 19:99-106.

Morized, J., P. Cruizilat, J. Chatenoud, P. Picot and P. Leclercq. 1984. Improvement of drought resistance in sunflower by interspecific crossing with a wild species *Helianthus argophyllus*. Methodology and 1 st results. Agronomie. 4:577-585.

Pustovoit, G. 1975. Selekcia podsolnecnika na gruppovoy immunitet metodom mejvidovoy gibridizacii. Proc. Podsolnecnik, M., "Kolos" p. 164-209.

Pustovoit, V. 1960. Mejvidovae i rjavchivoustoicivae gibrida podsolnecnika. Proc.: Otdalechennaia gibridizaciia rasteniy, Moskva. p. 376- 378.

Putt, E. D. and W. E. Sackston. 1957. Studies on Sunflower rust. I. Some sources of rust resistance. Can. J. Plant Science. 37:43-54.

Putt, E. D. and W. E. Sackston. 1963. Studies on sunflower rust. IV. Two genes R1 and R2 for resistance in the host. Can. J. Plant Science. 43:490-496.

Satsyperov, P. 1916. Opit skresthivaniia dvuh form podsolnecnika *H. annuus* x *H. argophyllus*. Tr. prikl. bot., gen. i sele., 9.

Serieys, H. and P. Vincourt. 1987. Characterization of some new cytoplasmic male sterility sources from *Helianthus* genus. Helia. 10:9-13.

Vannozzi, G. P., P. Megale, and E. Salera. 1990. *Helianthus* genus wild species interspecific hybrids: morphological and technological characters. Helia. 13(13):127-133.

Vranceanu, A. V., M. Iuoras, F. M. Stoenescu. 1986. A contribution to the diversification of cytoplasmic male sterility sources in sunflower. Helia . 9:21-25.